Kurlak, L.O. and Mistry, Hiten D. and Cindrova-Davies, Tereza and Burton, Graham and Broughton Pipkin, Fiona (2016) Human placental renin–angiotensin system in normotensive and pre-eclamptic pregnancies at high altitude and after acute hypoxia–reoxygenation insult. Journal of Physiology, 594 (5). pp. 1327-1340. ISSN 1469-7793

Alterations in human placental renin-angiotensin system in normotensive and pre-eclamptic pregnancies in women who delivered at high altitude and after acute hypoxia-reoxygenation insult.

Lesia O. Kurlak[1*], Hiten D. Mistry[1,2*], Tereza Cindrova-Davies[3], Graham J. Burton[3] & Fiona Broughton Pipkin[1]

[1] *Division of Obstetrics & Gynaecology, School of Clinical Sciences, University of Nottingham, City Hospital, UK*

[2] *Division of Hypertension, Department of Nephrology, Hypertension and Clinical Pharmacology, University of Bern, CH-3010 Berne, Switzerland.*

[3]*Centre for Trophoblast Research, University of Cambridge, UK*

** Authors made equal contribution to this study.*

Corresponding author's address: Dr. Lesia O. Kurlak

Division of Obstetrics & Gynaecology,

University of Nottingham,

1st Floor, Maternity Unit,

City Hospital, Hucknall Road,

Nottingham, NG5 1PB, UK

Lesia.kurlak@nottingham.ac.uk

Tel: +44(0)115 82 31955

Fax: +44 (0)115 82 31908

Running Title: The renin-angiotensin system responses to hypoxic insults

Key Words: renin-angiotensin system; hypoxia, placenta; hypoxia-re-oxygenation; hypoxia-inducible factors

Abstract (250 words)

A functioning placental renin-angiotensin system (RAS) appears necessary for uncomplicated pregnancy and is present during placentation, which occurs under low oxygen tensions. Placental RAS is increased in pre-eclampsia (PE), characterised by placental dysfunction, and elevated oxidative stress. We investigated the effect of high altitude (HA) hypoxia on the RAS and hypoxia-inducible factors (HIFs) by measuring mRNA and protein expression in term placentae from normotensive (NT) and PE women who delivered at sea-level or at above 3100m). A placental explant model of hypoxia-reoxygenation (hypoxia/reperfusion) was used to assess the impact of acute oxidative stress on the RAS and HIFs.

Protein expression of prorenin (P=0.049), prorenin receptor (PRR; P=0.0004), angiotensin receptors (AT1R; P=0.006) and (AT2R; P=0.002) were all significantly higher in NT women at altitude despite mRNA expression being unaffected. In contrast, mRNA expression of all RAS components was significantly lower in PE at altitude than at sea-level yet protein expression of PRR, angiotensinogen (AGT) and AT1R were all increased. The increase in transcript and protein expression of all the HIFs and NADPH oxidase 4 seen in PE compared to NT at sea-level was blunted at high altitude.

Experimentally-induced oxidative stress stimulated AGT mRNA (P=0.04) and protein (P=0.025). AT1R (r=0.77, P<0.001) and AT2R (r=0.81, P<0.001) mRNA were both significantly correlated with HIF-1β, whilst AT2R also correlated with HIF-1α (r=0.512, P<0.013).

Our observations suggest that the placental RAS is responsive to changes in tissue oxygenation: this could be important in the interplay between reactive oxygen species as cell-signalling molecules for angiogenesis and hence placental development and function.

Abbreviations: AGT, angiotensinogen; Ang II, angiotensin II; Ang IV, angiotensin IV; Ang (1-7), angiotensin-(1-7); AT1R, angiotensin type 1 receptor; AT2R, angiotensin type 2 receptor; $AT_{2A}R$, adenosine A2A receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HA, high altitude; HIF, hypoxia-inducible factor; NOX4, NADPH oxidase 4; H/R, hypoxia-reperfusion; NT, normotensive; MSU, midstream urine; PE, pre-eclampsia; PRR, prorenin receptor; RAS, renin–angiotensin system; ROS, reactive oxygen species; XO, xanthine oxidase**.**

Introduction

In the early 20th century, Sir Joseph Barcroft (Barcroft, 1946) famously postulated that the fetus may be considered as 'Everest *in utero*' because of its development in a hypoxic environment, probably only experienced at the Earth's highest point– the summit of Mt. Everest. Only relatively recently, arterial blood gases taken from climbers descending from this summit in 2007 have revealed just how astonishingly accurate this prediction turned out to be (West, 2009).

The transition from acute hypoxia to hypoxic adaptation may involve the renin-angiotensin system (RAS), which has been known as a regulator of systemic blood pressure and vascular function for many decades (reviewed in (Nguyen Dinh Cat & Touyz, 2011; Farag *et al.*, 2015) . Systemic RAS in male adults is activated by acute exposure to high altitude (HA) (Sun *et al.*, 2013), whereas exposure to chronic hypoxia, in rat models of human primary hypertension, the RAS is suppressed (Cervenka *et al.*, 2015). The RAS has also been well researched in the context of hypertensive disorders of pregnancy such as pre-eclampsia (PE) (Symonds *et al.*, 1975; Irani & Xia, 2011; Mistry *et al.*, 2013), a condition which has increased incidence at high altitude ((Palmer *et al.*, 1999; Keyes *et al.*, 2003).

However in recent years, it has become more evident that the RAS may play a significant role, not only in differing ways throughout pregnancy, but also in the early stages of placentation. Components such as the angiotensin receptors are expressed at very early stages of embryogenesis, when they may be responding to maternally-derived angiotensin II (AngII) (Pijacka *et al.*, 2012); (Tebbs *et al.*, 1999), in the placental syncytiotrophoblast and cytotrophoblast (Cooper *et al.*, 1999); (Williams *et al.*, 2010); prorenin and angiotensinogen (Marques *et al.*, 2011; Pringle *et al.*, 2011) as well as in the maternal decidua (Anton *et al.*, 2009). Both Ang II and the biologically-active fragment Angiotensin IV (3-8) (Ang IV) promote the invasion and proliferation of trophoblasts (Williams *et al.*, 2010) and angiogenesis in placental tissue (Pringle *et al.*, 2011), thus supporting the establishment of a placental vasculature.

Detailed knowledge on the process of implantation in humans is scarce, although the path from mother to the placenta must be established by the invasion of extravillous trophoblasts, starting from the tips of anchoring villi and intruding down into the inner third of the myometrium (Pijnenborg *et al.*, 2011); (Huppertz *et al.*, 2014). One fact is unequivocal: trophoblast invasion clearly correlates with oxygenation of the placenta (Huppertz *et al.*, 2014). Initial reorganisation of the syncytiotrophoblast layer and proliferation of the underlying cytotrophoblasts occurs in the first 5 weeks after implantation under oxygen tensions below 20 mmHg (Rodesch *et al.*, 1992);

(Jauniaux *et al.*, 2000): these oxygen tensions in other tissues would be considered 'hypoxic' but are physiologically normal for establishment of the placenta. Extravillous trophoblast invasion into the maternal tissue starts at higher partial pressures of 40-50 mmHg (Jauniaux *et al.*, 2000), after dissolution of endovascular trophoblast plugs within the spiral arteries enables the increase in maternal blood flow into the placenta. Direct *in vivo* oxygen measurements within the placental intervillous space are only available within the first 16 weeks (Jauniaux *et al.*, 2000), but indirect approximations indicate that in the second half of normal pregnancy, oxygen tensions range between 30-40 mmHg (Huppertz *et al.*, 2014).

In normotensive (NT) pregnancy, placental growth is stimulated by a number of factors including adenosine, which promotes angiogenesis, proliferation, inflammation and protection against oxidative stress (Hasko & Cronstein, 2004). We have previously established that there is a strong positive association between the RAS and adenosine in first trimester placental tissue (Kurlak *et al.*, 2015), when oxygen tensions are low (Rodesch *et al.*, 1992). The stimulation of adenosine is a response to hypoxia (Poth *et al.*, 2013) and therefore unsurprisingly placental adenosine receptor $A_{2A}R$ strongly correlates with HIF-1α (Kurlak *et al.*, 2015). Is the RAS acting as a signal transduction regulator for both oxygen sensing and angiogenesis? In other cell systems such as human fibroblasts cooperativity, between hypoxia- and angiotensin-induced proliferative responses has already been observed (Krick *et al.*, 2005), but a relationship between the RAS and low oxygen environment in the placenta has not yet been investigated.

In the pathological pregnancy condition of pre-eclampsia (PE), defined as *de novo* hypertension after 20 weeks gestation with significant proteinuria (Brown *et al.*, 2001; Tranquilli *et al.*, 2013), it has previously been suggested that the placental tissue is more hypoxic than in NT pregnancy. Since there are no direct measurements of intervillous oxygen tension beyond early second trimester, these assumptions have largely been based on evidence such as upregulation of global gene expression in PE showing similarities to *in vitro* and *in vivo* models of placental hypoxia (Soleymanlou *et al.*, 2005) and increases in hypoxia-inducible factors (HIFs) (Zamudio *et al.*, 2007); (Rajakumar *et al.*, 2008). These assumptions are being questioned in light of up-to-date evidence (Huppertz *et al.*, 2014), where placental metabolism in PE shows that there is no reduction in energy supplies as would be expected in chronic hypoxia (Bloxam *et al.*, 1987). It has been proposed (Hung & Burton, 2006) that the absolute oxygen concentration in the intervillous space may not be critically important, but rather fluctuations in oxygen tension that may lead to tissue oxidative stress as indicated by rapid degeneration of syncytium (Watson *et al.*, 1998). Each spiral artery delivers oxygenated blood to the centre of a placental lobule (Wigglesworth, 1969)

with fluctuations in intervillous blood flow during NT pregnancy. In pathological conditions such as PE, where there is reduced or absent dilation of the proximal end of the spiral arteries, flow may be even more intermittent. Sharp rises in oxygen tension within the lobule could induce excessive bursts of production of reactive oxygen species (ROS), which include free radicals such as superoxide anions, nitric oxide and hydroxyl radicals. Bursts of ROS production that overwhelm local antioxidant defences will result in oxidative stress. PE is characterised by both increased circulatory and placental oxidative stress (reviewed in (Goulopoulou & Davidge, 2015). There are several sources within the placental tissue that could generate ROS bursts, including mitochondria. These have been proposed as a source of the oxidative stress in PE, as demonstrated by swelling and loss of cristae, increased concentrations of malondialdehyde showing greater potential for lipid peroxidative damage (Wang & Walsh, 1998).

The formation of excessive amounts of free radicals and hence failure of mitochondrial reduction-oxidation balance have been tightly linked to dysregulation in the RAS (Vajapey *et al.*, 2014). One of the main peptides generated from angiotensinogen (AGT) susbstrate is Ang II, which binds with equal affinity to its type 1 (AT1R) and type 2 (AT2R) receptors (de Gasparo *et al.*, 2000). The binding of Ang II to AT1R activates NADPH oxidase (NOX), which leads to increased generation of cytoplasmic ROS. This Ang II-AT1R-NADPH-ROS signal triggers the opening of mitochondrial potassium (K_{ATP}) channels and mitochondrial ROS production in a positive feedback loop (Vajapey *et al.*, 2014). The maintenance of ROS balance means that the production of ROS needs to be matched with ROS scavenging mechanisms: Ang II has been implicated not only in activating ROS but also in decreasing the activity of scavenging enzymes (decreasing glutathione peroxidase, superoxide dismutase and catalase), thereby contributing even further to detrimental levels of ROS (Vajapey *et al.*, 2014).

Our group has already suggested a role for RAS components in the establishment of the placenta by demonstrating temporal changes in the Ang II receptors - AT1R, AT2R, AT4R (the receptor for bioactive Ang IV peptide) through gestation and a temporal relationship with the pro-oxidant enzymes – NADPH oxidase isoform (NOX4) and xanthine oxidase (XO) (Williams *et al.*, 2010). Furthermore, the placental RAS is altered in PE with AT1R being more highly expressed in the placenta from women with PE and AT1R expression alsonegatively correlating with infant birthweight and placental gluthathione peroxidase 3(Mistry *et al.*, 2013).

Pregnancy at HA gives a unique opportunity to investigate a natural model of PE. The incidence of PE is three-fold greater in areas above 2500 m (Moore *et al.*, 2011), where barometric pressure

falls from 760 mmHg at sea level where the PO_2 of ambient air is 159 mmHg, to a barometric pressure of 537 mmHg at 3000 m. At such altitude the birthweights are smaller by an average of 102 g/1000 m elevation, despite placental weights being comparable (Yung *et al.*, 2012). On the basis of these considerations, we set out to investigate in this study (i) whether tissue-based RAS is activated by HA hypoxia by examining placentae from NT women who have delivered at HA compared to those who resided at sea-level before and throughout the pregnancy; (ii) to determine whether there is a further effect of PE on the RAS in placentae from PE women at altitude. In addition (iii) we have tested the theory that oxidative stress induced by hypoxia/reperfusion (H/R) injury might be the activator of the RAS as opposed to hypoxia *per se,* by culturing term placental tissue explants *in vitro,* under differing conditions of hypoxia and oxidative species.

Methods

Ethical approval

All samples were collected following provision of informed, written consent conforming to the standards set by the Declaration of Helsinki, and all procedures were approved by the local ethics committee (Hospital Ethics Committee of the Nottingham University hospitals, University of Colorado Health Sciences Center (Aurora, CO, USA) and University College Hospital London (London, UK), and the Cambridge Local Research Ethics Committee).

Subjects, selection criteria and sample collection

NT control and PE placentae were collected from women at both sea-level and HA. PE was stringently defined as per the International Society for the Study of Hypertension in Pregnancy guidelines of a systolic blood pressure of 140 mm Hg or more and diastolic pressure (Korotkoff V) of 90 mm Hg or more on 2 occasions after 20 weeks gestation in a previously NT woman and proteinuria ≥300 mg/L, ≥500 mg/day or ≥2+ on dipstick analysis of midstream urine (MSU) if 24-hour collection result was not available (Brown *et al.*, 2001). Exclusion criteria included smoking, renal disease, cardiac disease, diabetes, chronic hypertension, pregnancy-induced hypertension, or any other complications of pregnancy, and known risk factors for these conditions. All placentae were from term deliveries, either by elective Caesarean section (CS) or spontaneous vaginal delivery (SVD). The HA placentae were collected at St. Vincent's General Hospital (Leadville, CO, USA; 3100 m) from stable, but recently migrant, populations of European descent. The sea-level placentae were collected at the Rosie Hospital (Cambridge, UK) and, Nottingham University Hospitals (Nottingham, UK) and University College Hospital (London, UK). By the very nature of the collections, the subject numbers differed in the various parts of the study and will be specified with the results.

Samples were obtained using a systematic random system by which the placenta was divided into 5 areas (Hung *et al.*, 2001a). Two full-thickness samples were taken from each area, washed in phosphate buffered saline (PBS) to remove excess blood contamination, snap-frozen in liquid nitrogen within 10 min of delivery, and then maintained at −80°C until further processed. Samples for immunohistochemistry were immediately submersed in formalin to be fixed for 12–24 h. After being treated with 70% ethanol for 24 h, placental samples were further dissected before being embedded in paraffin.

Acute hypoxia experiments

Term placentae were obtained from NT pregnancies immediately after elective CS delivery before onset of labour as previously described (Hung *et al.*, 2001b);(Hung *et al.*, 2002). Briefly, villous samples (~40-50 mg wet weight) were taken midway between the chorionic and basal plates, from 5-7 lobulae free of visible infarction, calcification, hematoma, or tears. After a brief wash in cold PBS, samples were placed in culture medium (Medium-199 with 25 mmol/L HEPES, Earles' salts, and L-glutamine; Life Technologies Ltd., Paisley, UK) equilibrated with 95% N_2/5% CO_2 (BOC, Guildford, UK) in sealed glass bottles, and transferred to the laboratory on ice for individual experiments.

After dissection, 6 pieces of villous tissue (~5 to 10 mg) were cultured in individual Costar Netwell (24-mm diameter, 500-μm mesh; Corning) supports in 1.5 mL of culture medium. A gas mixture of 10% O_2/85% N_2/5% CO_2 was used as the normoxic condition, and the dissolved oxygen tension in the medium was 45-62 mm Hg. Conditions for hypoxia and H/R were established as previously described (Hung *et al.*, 2001b). For H/R experiments, villous tissues were cultured under hypoxic conditions (PO_2 in medium = 12 - 16 mm Hg) at 37°C for 3 hours, then transferred to medium equilibrated with air/5% CO_2 in a separate humidified chamber continuously flushed with air/5% CO_2 for an additional 4 hours (PO_2 in medium = 143 - 160 mm Hg). In control experiments, villous tissues were kept under either normoxic conditions throughout the 7-hour period with a change of medium at 3 hours. Additional samples were incubated under normoxic conditions with either 1 mM hydrogen peroxide (H_2O_2) or vitamins C and E (1 mM for both).

RNA extraction and cDNA synthesis

Total RNA was extracted from a known amount of placental tissue (~100 mg) using QIAzol lysis reagent (Qiagen, Crawley, UK) as previously described (Mistry *et al.*, 2011). RNA concentration and purity were verified spectrophotometrically using the Nanodrop ND-1000 (Nanodrop Technologies, Labtech, Ringmer, UK); all of the samples had an A260/A280 ratio >1.96. RNA (1 μg) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen, Crawley, UK) in a Primus96 thermocycler (Peqlab Ltd, Fareham, UK).

Quantitative real time Polymerase Chain Reaction (PCR)

Real-time PCR was carried out using SYBR Green chemistry (2x QuantiFast SYBR Green, Qiagen, Crawley, UK) on a ABI7500 Fast (Life Technologies,UK) using the primers detailed in Table 1. Melt-curve analysis was performed to confirm the presence of one single product and non-template controls were run to assess contamination. The standard curve method was used to

quantify gene expression. Abundance data for the genes of interest were expressed following normalisation using GeNORM (http://medgen.ugent.be/~jvdesomp/genorm/), with 3 stably expressed housekeeping genes, suitable for human placental samples: GAPDH, β-2-microglobulin and β-actin (Murthi *et al.*, 2008); (Kurlak *et al.*, 2013) and expressed normalised to copy number.

Immunohistochemical staining of RAS components and markers of hypoxia/oxidative stress

Placental protein expression was analysed by immunohistochemistry. Serial sections of placental tissue were cut (5 μm) in the same orientation from paraffin-embedded tissue blocks (Sledge Microtome, Anglia Scientific, Norwich, UK) and mounted onto Superfrost plus glass microscope slides (Menzel-Glaser, Braunschweig, Germany). Before use, sections were dewaxed by immersion in xylene followed by rehydration in descending concentrations of alcohol (5 minutes each).

Immunohistochemical staining was performed using the Dako Envision staining kits (Dako Ltd Germany). Eight rabbit and 2 mouse antibodies were used for immunostaining of paraffin-embedded placental sections. The optimal dilutions and details of all antibodies used are shown in Table 2. Heat-induced epitope retrieval was achieved by heating in a citrate buffer (pH 6.0) using a microwave oven for 15 minutes (800 W), followed by incubation for 30 minutes in normal goat serum (Sigma-Aldrich, UK) to block nonspecific binding; slides were then incubated with primary antibodies overnight at 4°C. A negative control was performed for each test section by incubation with mouse or rabbit IgG as appropriate. Sections were dehydrated and cleared in ascending concentrations of alcohol and xylene before mounting in DPX (BDH, Poole, United Kingdom).

For analysis of placental sections, digital images of 5 randomly selected, high-power (×400 magnification) fields were captured on NIS-Elements F2.20 microscope (Nikon UK Ltd, UK). Protein expression was semi-quantitatively assessed (H-score) at x200 magnification (Nikon Eclipse II microscope) by two blinded observers (LOK & HDM), as described previously (Mistry *et al.*, 2013). Between-observer agreement for H-scoring was excellent (kappa 0.97). A visual check was also performed to ensure accurate discrimination of immunolabeled regions.

Statistical analysis

All tests were performed using SPSS for Windows version 22 and GraphPad PRISM, version 6. Summary data are presented as means ± standard deviation (SD) or median and interquartile range

(IQR) as appropriate, the one-way ANOVA or Kruskal-Wallis tests followed by Gabriel's or Mann-Whitney U *post hoc* tests were used depending on the distribution of the data, after testing using the Kolmogorov-Smirnov test. Correlations between the parameters were tested with a Spearman's Rank test. The null hypothesis was rejected where $P<0.05$. The data from the *in vitro* culture experiments were evaluated using repeated measures analysis.

Results

A. *Ex vivo* data

(i). *Placental RAS in NTwomen at HA*

mRNA measurements were available from 9 placentae collected at sea-level (SVD: n = 4; CS: n = 5) and 8 at HA (SVD: n = 4; CS: n = 4). For the protein expression study, 16 placentae were collected at sea level n=16, (SVD: n=9; CS: n=7), and 8 at HA (SVD: n = 4; CS: n = 4).

The mRNA expression of all RAS components was not significantly affected by altitude in NT women, although variation was higher for all components at HA (Fig. 1A). In contrast, the protein expression of prorenin ($P = 0.049$), prorenin receptor (PRR; $P = 0.0004$) and both AT1R ($P = 0.006$) and AT2R ($P = 0.002$) were all significantly higher in NT placentae from HA (Fig. 1B), although expression of AGT was not different ($P > 0.1$). All proteins were confined to the outer layer of the villous syncytiotrophoblast. The ratio between prorenin and its receptor, PRR also rose significantly ($P = 0.008$), while that between the AT1R and AT2R was unchanged ($P > 0.8$). The type of delivery did not influence protein expression of any of the RAS components ($P > 0.2$ to > 0.7) and therefore we did not consider mode of delivery as a confounder in further analyses.

(ii). *Placental RAS in women with PE at HA*

mRNA measurements were available from 19 placentae collected at sea-level (n = 11) and at HA (n = 8). Samples analysed for proteins included 6 collected at sea level and 5 at HA.

The mRNA expression was significantly lower in PE women at HA, for all RAS components (Fig. 2A) except the AT1R. By contrast with samples from NT women (Fig. 1A), the variability in mRNA expression was also markedly less. However, the protein expressions of PRR, AGT and AT1R were all increased in PE at altitude (Fig. 2B) although this only reached statistical significance for AGT and AT1R.

Placental HIFs and NOX4 in NT and PE at altitude

In the HA NT pregnancies, placental mRNA expression of all HIFs and NOX4 was unaffected by altitude (Fig. 3A). However, the protein expression of all the HIFs: HIF-1α ($P < 0.001$); HIF-1β

($P < 0.001$); HIF-2α ($P = 0.04$) as well as NOX4 ($P = 0.001$) was significantly raised in high altitude pregnancies (Fig.3B). The enzyme XO, was unaffected by altitude (data not shown). Immunohistochemical staining was localised to the syncytiotrophoblast cells and around the fetal vessels of the placental villi.

The effect of altitude on the pattern of expression differed in the placentae from women with PE. Whereas there was no effect of altitude in the NT women, the mRNA expression of HIF-1α, HIF-1β and NOX4 mRNA expression were all lowered at HA compared to the sea-level PE women ($P < 0.001$ for all; Fig. 3A). When comparing placentae from NT and PE women at sea-level, HIF-1α and NOX4 mRNA expression was markedly higher in the PE compared to NT ($P = 0.01$ and $P = 0.001$ respectively). At HA, HIF-2α mRNA expression was lower in PE than NT ($P = 0.003$; Fig. 3A).

Protein expression was equally affected by altitude in the PE women: HIF-1α ($P < 0.01$); HIF-1β ($P = 0.03$); HIF-2α ($P = 0.017$) as well as NOX4 ($P = 0.004$) were significantly lower in high altitude pregnancies in women with PE (Fig. 3B). Xanthine oxidase protein expression was not markedly altered (data not shown).

In HA samples, protein expression of HIF-1α ($P = 0.001$); HIF-1β ($P = 0.001$); HIF-2α ($P = 0.029$) and NOX4 ($P = 0.019$) was significantly lower in women with PE as compared to NT women (Fig. 3B).

B. *In vitro* data

(i) In vitro explant culture under experimental oxidative stress conditions

Due to the nature of the explant methodology, sample numbers in each of the treatment groups were 4-6 depending on availability of sufficient quality tissue.

Experimental H/R treatment significantly only increased the mRNA expression of AGT ($P = 0.04$; Fig. 4A) compared to normoxic conditions. Protein expression followed the same pattern with only AGT enhanced ($P = 0.025$) with H/R treatment (Fig. 4B). Treatment with H_2O_2 under normoxic oxygen concentrations, and normoxia with added free radical scavengers (vitamins C and E), both, had no effect on AGT mRNA, although both H_2O_2 and H/R were associated with significantly-increased AGT protein expression (Fig. 4B). No other components of the RAS were significantly altered by any of the treatments.

The HIFs and antioxidant enzymes were also measured in these experiments, to establish whether hypoxia was being sensed within this model. There was no observable effect of these experimental conditions on either mRNA expression or protein expression, of any of the HIFs, NOX4, or XO. Interestingly, we identified strong positive correlations in gene expression between HIF-1β and both angiotensin receptors AT1R ($r=0.77$, $P<0.001$) and AT2R ($r = 0.81$, $P < 0.001$), but only AT2R also correlated with HIF-1α ($r = 0.512$, $P < 0.013$; Fig. 5).

Discussion

Our aim was to investigate the effects of oxidative stress on the RAS in the human placenta, whether associated with the lower pO_2 of HA chronic hypoxia, with the elevated tissue and circulatory chronic oxidative stress, which characterises PE, or with the acute experimental oxidative stress induced *in vitro* by a H/R insult. Our novel results demonstrate, for the first time that, components of the RAS are activated under these conditions, which components and to what extent is dependent on the nature of the insult.

At altitude, the picture in these placentae from women chronically exposed to hypoxia is one of a generalised, significant increase in activity of the RAS, which, to our knowledge, has not been reported previously. The rise in PRR is statistically highly-significant, and the binding of (pro) renin to this receptor induces a 4-fold increase in its catalytic efficiency in the generation of angiotensin I (Ang I) from AGT (Nguyen *et al.*, 2002). Thus, changes in PRR expression even without changes in AGT expression can have a substantial effect on the generation rate of Ang I, and hence Ang II. Furthermore, our results from the *in vitro* H/R model show a significant increase in both mRNA and protein expression of AGT supporting our premise that this element of the renin-angiotensin axis may be instrumental in 'tuning' the regulation of placental RAS in a redox environment. When oxidised, AGT is reacted with renin in the presence of the PRR, there is a 4-fold increase in the generation of Ang I (in addition to the 4-fold increase induced by the binding of prorenin to PRR), which does not occur if reduced AGT is reacted (Zhou *et al.*, 2010). The hypoxia of altitude might be enough to increase the ratio of oxidised: reduced AGT and further stimulate the system. This appears to be corroborated by the rise in AGT observed after an acute hypoxic insult in the experimental model. Our observations suggest that this increase in AGT is not simply a reaction to lower oxygen tension *per se* but potentially relates to the increased oxidative stress induced by this model (Cindrova-Davies *et al.*, 2007). We could only examine total AGT expression, as it is not yet possible to distinguish the oxidised and reduced forms immunohistochemically; total expression was not different at the two altitudes.

Low concentrations of endogenous ROS are important in the activation of signalling pathways, which relate to hypoxia adaptation, such as genes involved in angiogenesis (e.g. vascular endothelial growth factor;VEGF), where the promoter region has a binding site for H_2O_2 (Oshikawa *et al.*, 2010), which is essential for full activation of VEGF receptor 2 (VEGFR2). Accumulating evidence points towards the existence of direct interactions between Ang II and VEGF; a recent study has demonstrated that AT2R mediates the synergistic effect of a combination of Ang II and VEGF-A to promote differentiation of bone marrow-derived

mesenchymal stem cells into endothelial cells (Ikhapoh *et al.*, 2015). These authors suggest that AT2R could be critical to repair and regenerate damaged vascular endothelium. In other cell systems such as mouse Lewis lung carcinoma cells, hypoxia appears to upregulate ACE, Ang II, and AT1R. Most intriguingly, this regulation is diphasic: in the early phase, after 6 hrs hypoxia, AT1R protein is decreased, whilst AT2R protein is increased; yet if hypoxia is continued up to 24 hrs, this effect is reversed, with AT1R protein significantly increased and AT2R decreased (Fan *et al.*, 2014). Employing specific pharmacological inhibitors, these authors have concluded that this diphasic response may be due to a bi-directional control mechanism: hypoxia enhances the expression of AT1R and AT2R via increased Ang II, but elevated AT2R inhibits AT1R during the early stage and as hypoxia continues, elevated AT1R then inhibits AT2R expression. This may explain why neither the AT1R nor the AT2R were significantly altered in our H/R model. Nevertheless, the strong association, observed in this study, between both types of angiotensin receptors and the oxygen-sensing units (HIF-1β and HIF-1α) adds credence to the idea that these receptors are involved in the oxygen sensing mechanism of the syncytiotrophoblasts. AT2R may have a protective role in the repair of oxygenation-induced damage (Namsolleck *et al.*, 2014), as seen in ischaemic stroke (McCarthy *et al.*, 2009), myocardial ischaemic injury (Parlakpinar *et al.*, 2011; Park *et al.*, 2013) and neurological pathologies (Sumners *et al.*, 2013).

By the same token, prorenin has also been shown to stimulate proliferation of retinal endothelial cells to an equivalent degree as that induced by VEGF and independently of Ang II (Zhu *et al.*, 2015). In pancreatic cancer cells, Ang II induces VEGF, through AT1R and ERK1/2 signalling (Anandanadesan *et al.*, 2008). Studies in AGT-knockout mice report blunted hypoxia-induced endothelial progenitor cell function indicating that AGT is required for hypoxia-induced vasculogenesis (Choi *et al.*, 2014).

Since hypoxia stimulates the release of ROS, we postulated that the low oxygen partial pressure at altitude (HA hypoxia) would amplify the oxidative stress of PE, through increased production of ROS, and thus augment the stimulation of the RAS already observed in PE at sea-level. However, this stimulation did not occur: whereas mRNA expression of the RAS was unaffected in NT women at altitude (Fig. 1A), mRNA expression of the RAS was actually reduced in PE at altitude (Fig. 2A). However, translation followed a very different pattern, showing increased translation (protein expression) in a majority of components of the RAS, in both NT and PE women at HA.

Concurrent analysis of the mRNA expression of the HIFs (Fig. 3) revealed that there were no detectable differences between sea-level and HA in the NT women; this agrees with previously published data (Tissot van Patot *et al.*, 2004). In contrast, the increases in HIF-1α, and NOX4 observed in PE compared to NT women at sea-level were not observed in PE at HA; indeed, mRNA for HIF-1α, HIF-1β and NOX4 were significantly lower in PE women at HA. Our protein data (Fig. 3) expand the study of Zamudio et al (Zamudio *et al.*, 2007), which reported increases in the HIF-1α protein expression at HA in NT women. What is completely novel in our study is the observation that at HA, placental responses to oxygen deprivation in women with PE are completely blunted, as demonstrated by the lower mRNA and protein parameters of all the HIFs. Despite the low sample numbers, these observed differences are striking. This certainly begs the question – do pregnant women who develop PE at HA have an adaptive response to hypoxia that differs to that of NT women at HA? Rajakumar et al (Rajakumar *et al.*, 2008) suggest that impairment of protein degradation, rather than increased synthesis causes inadequate oxygen-dependent reduction of HIF-1α protein in PE and is supported by the fact that, in early-onset PE placentae at least, there is decreased expression of the prolyl hydroxylases, PHD1 and PHD2 (Rolfo *et al.*, 2010). Hence, molecular events leading to the formation of the HIF-1α:VHL-ubiquitin ligase complex (Semenza, 2007), as well as proteasomal trypsin, chymotrypsin, and peptidyl glutamyl-like activities may be the focus of adaptation. In well-oxygenated cells, PHDs hydroxylate the HIF-1α subunits, thereby targeting them for proteosomal degradation and resulting in a half-life of less than 5 minutes (Wang *et al.*, 1995). PHDs are the key enzymes triggering HIF-1α desensitization, a feedback mechanism required to protect cells against necrotic cell death and thus to adapt them to chronic hypoxia (Ginouves *et al.*, 2008). Whereas humans living at low altitudes ordinarily maintain low levels of HIF-1α and HIF-2α, through constitutive hydroxylation and degradation, upon ascent to high altitude, attenuated PHD2 hydroxylase activity due to hypoxia, leads to increased levels of HIF-1α and HIF-2α. Even so, the Tibetan population shows the strongest support for the hypothesis that multiple genes in the HIF pathway can be reconfigured in response to chronic hypoxia (Bigham & Lee, 2014); (Lorenzo *et al.*, 2014): the most convincing candidates for this reconfiguration being PHD2 and HIF-2α. Tibetans are the first humans in whom a hypo-responsive HIF system has been demonstrated, and may represent an evolutionary resetting of the HIF system to operate within a hypoxic environment (Petousi *et al.*, 2014). However, this may differentiate populations long-acclimatised to life at high altitude from those relatively-recently arrived, such as the inhabitants of Colorado included in this study.

As noted in the Introduction, spiral artery remodelling is a period of H/R insult. In order to explore the possibility that oxidative stress and not hypoxia *per se* is the driving force behind the activation of the RAS at HA and in PE, we used the fully-validated experimental model of H/R. In this model, increased oxidative stress is demonstrated by the increase in heat shock proteins Hsp27 and Hsp90, lipid peroxidation and formation of peroxynitrite (Cindrova-Davies *et al.*, 2007), localised mainly to the syncytiotrophoblast. The addition of the ROS scavengers, vitamins C and E significantly reduces markers of oxidative stress in the H/R-treated samples. Under these induced acute conditions of oxidative stress, our study reveals a more specific stimulation of the RAS, as seen by increased mRNA and protein expression of AGT only (Fig. 4). The addition of vitamins C and E diminishes this increase, although this is not statistically significant, possibly due to the small sample sizes. Nonetheless, this reversal may suggest that the production of AGT is specifically sensitive to the redox environment (see also above). The fact that other RAS components were not increased by the acute oxidative stress of these experimental conditions implies that their generalised stimulation at HA may be a response to the chronically-reduced oxygen tensions. This is supported by the fact that in a mouse model of alveolar hypoxia, such as may occur in pulmonary diseases (e.g chronic obstructive pulmonary disease), the RAS is stimulated by the low PO_2 (Gonzalez & Wood, 2010) and it is the activation of NADPH oxidase by the RAS that leads to an inflammatory response. However, if the animals remain under hypoxic conditions for several days, the inflammation resolves and exposure to lower PO_2 does not elicit further inflammation, suggesting that the vascular endothelium has "acclimatised" to hypoxia (Gonzalez & Wood, 2010). Furthermore, in human lung fibroblast cells, hypoxia directly activates the RAS to induce collagen type I expression, mediated by NF-κB signalling, which in turn activates AT1R expression (Liu *et al.*, 2013).

Previously, using the different methodology of Western blotting to measure protein in term villi, H/R was found to stimulate HIF-1α in this model (Cindrova-Davies, 2009). Using the semi-quantitative method of immunohistochemical scoring, we did not detect this stimulation. Likewise, use of H_2O_2 to mimic the effect of oxygen in acutely lowering HIF-1α, did not have this effect in our placental explants. This discrepancy may be due to differences in methodological sensitivity.

Hypoxia cannot be merely defined by just the prevailing oxygen tension (Cindrova-Davies *et al.*, 2015), but must be related to the metabolic demands of the tissue. As Barcroft himself said "oxygen -is- but one of the proximate principles of nourishment" (Young, 1992). The placenta is an extremely metabolically active tissue (Carter, 2000; Illsley *et al.*, 2010). Oxygen sensing is of

fundamental importance to all cells as oxygen consumption is required for ATP generation (Guzy & Schumacker, 2006). In tissue such as the placenta, which is exposed not only to altered oxygen concentrations, and H/R insults during development, but also within its own lobule structures, oxygen sensing is critical in order to stimulate gene expression and transcription of genes relating to growth processes, including angiogenesis. An ability to control oxygen sensing mechanisms using molecules, which do not disrupt oxygen consumption, would have a profound impact on a diverse range of pathologies. We have here described such a potential system.

Competing interests: No conflict of interest for any authors.

Acknowledgements

Author contributions: Conception and design of the experiments (FBP & GJB); Collection, assembly, analysis and interpretation of data (HDM, LOK & TC-D); Drafting the article or revising it critically for important intellectual content (LOK, HDM, TC-D, FBP & GJB)

We thank Dr. Martha Tissot van Patot for supplying high altitude samples from Colorado and Mr.Yosef Mansour for technical assistance with qPCR .

Sources of Funding: HDM is supported by an ERA-EDTA Fellowship (ERA LTF 137-2013).

Table 1. Details of primers used in qPCR.

Gene	Accession number	Primers	Length (bp)
PRR	NM	5’ cctcattaggaagacaaggactatcc 3’ 5’ gggttcttcgcttgttttgc 3’	50
Prorenin	NM	5’ ccacctcctccgtgatcct 3’ 5’ gcggatagtactgggtgtccat 3’	46
AGT	NM_000029	5` ccccagtctgagatggctc 3` 5` gacgaggtggaaggggtgta 3`	117
AT1R	NM_004835	5’ ggctattgttcacccatgaagt 3’ 5’ tgggactcataatggaaagcac 3’	177
AT2R	NM_000686	5’ tatggcctgtttgtcctcattg 3’ 5’ ccattgggcatatttctcaggt 3’	115
HIF1α	NM_ 181054	5’ atccatgtgaccatgaggaaatg 3’ 5’ tcggctagttagggtacacttc 3’	125
HIF1β	NM_ 001197325	5’ tgactcctgttttgaaccagc 3’ 5’ ctgctcacgaagtttatccacat 3’	95
HIF2α	NM_ 001430	5’ ggacttacacaggtggagcta 3’ 5’ tctcacgaatctcctcatggt 3’	79
NOX4	NM_016931.3	5’ tgaactatgaggtcagcctctg 3’ 5’ aagcttgtatggtttccagtcat 3’	107
GAPDH	NM_002046.3	5’ ggaagcttgtcatcaatggaa 3’ 5’ tggactccacgacgtactca 3’	102
B-Actin	NM_001101.2	5’ ccaaccgcgagaagatga 3’ 5’ ccagaggcgtacaggatag 3’	97
B2M	NM_004048.2	5’ cttatgcacgcttaactatcttaacaa 3’ 5’ taggagggctggcaacttag 3’	127

Table 2. Product descriptions and concentrations for immunohistochemistry

	Product name/Description	**Concentration (mg/mL)**
PRR	Anti- ATP6AP2 - *Sigma Prestige,* HPA003156. Rabbit polyclonal	1.8
Prorenin	Anti-prorenin –*Abcam,* ab82450. Mouse monoclonal	8.35 (1 in 200)
AGT	Anti-AGT – *Sigma Prestige,* HPA0031557. Rabbit polyclonal	0.26
AT1R	Anti-AT1R – *Abcam,* ab9391. Mouse monoclonal	80
AT2R	Anti-AT2R - *Abcam,* ab19134 Rabbit ploYclonal	5.2
HIF1α	Anti- HIF1α – *Sigma Prestige,* HPA0031557 Rabbit polyclonal	1 in 200
HIF1β	Anti- Arnt – *Aviva Systems Biology,* ARP30979. Rabbit polyclonal	1:1000
HIF2α	Anti-EPAS1 - *Aviva Systems Biology,* ARP32253. Rabbit polyclonal	1:200
NOX4	Anti-NOX4 - *Abcam,* ab133303. Rabbit polyclonal	1:500
XDH	Anti-XDH - *Aviva systems biology* OAAB05869. Rabbit polyclonal	1:500

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Figure Legends

Figure 1: A) Normalised mRNA expression (copy number) of all RAS components in placentae from normotensive controls residing and delivering at sea level (n = 9) or at high altitude (n = 8). B) Placental protein expression and localisation of RAS components from normotensive controls residing and delivering at sea level (n = 16) or at high altitude (n = 8). Boxplots represent median [interquartile range]; *P<0.05; **P<0.001; ***P<0.0001. Positive staining was localised mainly in syncytiotrophoblasts (black arrows). All photomicrographs taken at x400 magnification; positive staining is shown in brown. PRR: prorenin receptor; AGT: angiotensinogen; AT1R/2R: angiotensin receptors type 1 and 2.

Figure 2: A) Normalised mRNA expression (copy number) of all RAS components in placentae from pre-eclamptic women residing and delivering at sea level (n = 11) or at high altitude (n = 8). B) Placental protein expression and localisation of RAS components from pre-eclamptic women residing and delivering at sea level (n = 6) or at high altitude (n = 5). Boxplots represent median [interquartile range]; **P<0.001; ***P<0.0001. Positive staining was localised mainly in syncytiotrophoblasts (black arrows). All photomicrographs taken at x400 magnification; positive staining is shown in brown. PRR: prorenin receptor; AGT: angiotensinogen; AT1R/2R: angiotensin receptors type 1 and 2.

Figure 3: A) Normalised mRNA expression (copy number) of all hypoxia-inducible factor (HIF) components and NADPH oxidase (NOX4) in placentae from normotensive controls (NT) and pre-eclampsia (PE) residing and delivering at sea level or at high altitude. B) Placental protein expression and localisation of HIF components and NOX4 from NT and PE women residing and delivering at sea level or at high altitude. Boxplots represent median [interquartile range]; *P<0.05; **P<0.001; ***P<0.0001. Positive staining was localised mainly in syncytiotrophoblasts (black rarrows), with some staining around fetal vessels (red arrows). All photomicrographs taken at x400 magnification; positive staining is shown in brown.

Figure 4: Placental angiotensinogen A) mRNA and B) protein expression of term placental explants (n = 4-6) following incubation in normoxic conditions; following hydrogen peroxide (H_2O_2); after hypoxia/reperfusion (H/R) or with added free radical scavengers vitamins C and E (vitamins). Data analysed using repeated measures analysis and are represented at mean ± standard deviations; **P<0.001. Positive staining was localised mainly in syncytiotrophoblasts (black arrows). All photomicrographs taken at x400 magnification; positive staining is shown in brown. AGT: angiotensinogen.

Figure 5: Scatter plots demonstrating associations in placental mRNA expression of AT1R, AT2R, HIF-1β and HIF-α. HIF-1β strongly correlated with both AT1R (r= 0.77; *P*<0.001) and AT2R (r = 0.81; *P* <0.001) under all oxygenation conditions. AT2R also correlated with HIF-1α (r = 0.512; *P* < 0.01).